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Review

The relevance of citrullinated vimentin in the production of antibodies against citrullinated proteins and the pathogenesis of rheumatoid arthritis

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Abstract

Antibodies against citrullinated proteins (ACPAs) are highly specific for RA. Since the discovery of these antibodies, several of studies that focused on the presence and identity of citrullinated proteins in the joints of RA patients have been carried out. The best-known antigens that bind ACPAs are citrullinated filaggrin, Type II collagen (CII), α-enolase, fibrinogen and vimentin. This review compares citrullinated filaggrin, CII, α-enolase and fibrinogen with vimentin in their contribution to ACPA triggering, and gives an overview of the literature in which the role of citrullinated and non-citrullinated vimentin in the onset of ACPA production and the pathogenesis of RA is discussed.

Key words: Rheumatoid arthritis, Citrullinated vimentin, Citrullinated fibrinogen, Citrullinated filaggrin, Type II collagen, α-enolase, Anti-Sa, Anti-mutated citrullinated vimentin, Immune complexes.

REVIEW

Introduction

Antibodies against citrullinated proteins (ACPAs) are considered as very specific markers for RA (94–99%) (reviewed in [1]; [2–6]) (Table 1). Moreover, citrullinated proteins and antibodies against these citrullinated proteins are known to have an important role in the pathogenesis of RA (reviewed in [7]). This diagnostic marker is also found to be associated with the HLA shared epitope (SE), confirming their specificity for RA [8]. Other promising findings of these antibodies are their presence in serum before disease onset and their correlation with disease severity [7, 9]. Until now, the best-known potential triggers for ACPA production have been filaggrin, Type II collagen (CII), α-enolase, fibrin(ogen) and vimentin (reviewed in [10, 11]).

In this review, we compare the ability of these antigens to initiate ACPA production and focus on the role of citrullinated vimentin in the pathogenesis of RA and the early stage of ACPA production.

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Comparison of (pro)filaggrin, CII, α-enolase, fibrinogen and vimentin in their ability to trigger ACPA production

Localization in the joint

An important difference between (pro)filaggrin and the other proteins (CII, α-enolase, fibrinogen and vimentin) is their presence in the joint. (Pro)filaggrin has not been found in the joints of RA patients, whereas CII, α-enolase, fibrin(ogen) and vimentin, have been detected in large amounts [18–22]. Since citrulline-reactive autoantibodies are synthesized locally by plasma cells in the pannus, the trigger for ACPA production must be a (self-)antigen present in the inflamed joint [23, 24]. Therefore, one could presume that citrullinated pro-filaggrin is not the autoantigen that drives the ACPA response [23]. Since citrullinated CII, α-enolase, fibrin(ogen) and vimentin are detected in the joints of RA patients [18, 19], they are considered as more relevant candidates to trigger the ACPA production.

Although filaggrin is not present in the joint, Masson-Bessiere *et al.* [25] found that anti-filaggrin autoantibodies (AFAs) could bind proteins from the joint such as citrullinated α- and β-fibrin chains, which are found in the synovium of RA patients. AFAs are, therefore, postulated as antibodies reactive to citrullinated epitopes from a cross-reactive protein [17, 25]. This was confirmed by Baeten

Table 1 Comparison of the sensitivity and specificity for anti-CCP titre, anti-Sa titre and anti-MCV titre

	Sensitivity, %	Specificity, %	Reference	Total number of patients/controls	Number of RA patients
Anti-CCP	75.4	97.3	[1]	264	118
	66.4	98.3	[2]	237	119
	70.1	98.7	[3]	467	164
	92.8	93.7	[5]	78	56
	72.4	96.1	[6]	479	170
	41	91	[27][a]	238	106
	57.9	96	[65][b]	373	273
	48	98	[74][c]	175	63
Anti-Sa	43	99	[49]	482	206
	31	98	[51]	277	67
	22	98	[27][a]	238	106
Anti-MCV	75.6	91.5	[2]	237	119
	69.5	90.8	[3]	467	164
	74.1	79	[6]	479	170
	70.7	95	[65][b]	373	273
	54	91	[74][c]	175	63
	84	87	[64]	292	92[d]

[a]Patients with recent onset of RA (peripheral joint synovitis of <12 months duration). [b]At the time of diagnosis [65]. [c]Within 3 months of the onset of synovitis. [d]Sixty-eight RA patients were evaluated to determine anti-MCV sensitivity; 92 RA patients were evaluated to determine anti-MCV specificity.

et al. [26] who found by means of double IF that AFA reactivity colocalized with anti-citrulline reactivity in the synovium but not with monoclonal AFAs that recognized both filaggrin and pro-filaggrin. This cross-reactivity of AFAs with other citrullinated proteins was explained by Schellekens *et al.* [17], who suggested that citrulline is essential for the antigenic properties of proteins recognized by RA-specific antibodies. Although anti-keratin antibodies (AKAs), anti-perinuclear factors (APFs), AFAs, ACPAs and antibodies against citrullinated vimentin show certain cross-reactivity and preferentially recognize citrullinated antigens, Goldbach-Mansky *et al.* [27] noted that the modest degree of concordance between them in individual patient sera suggests that it is unlikely that a single antigen is responsible for all these reactivities. However, it should be noted that the ELISA technique used by Goldbach-Mansky *et al.* [27] is a good technique to detect cross-reactivity, but is not appropriate to identify the original antigen.

Intra- and extracellular proteins

Despite the fact that ACPAs are specific for RA, several studies have shown that citrullinated proteins in the inflamed synovium are not specific for RA. They are rather associated with inflammation [28, 29]. In murine models of RA (CIA and streptococcal cell wall-induced arthritis), several citrullinated proteins including fibrin could be detected [30]. However, antibodies against cyclic citrullinated proteins (anti-CCP) could not be detected in these mice [30]. Therefore, we can conclude that ACPA production is not merely due to the presence of citrullinated proteins in the joint [28, 29, 31].

Intracellular citrullinated proteins, on the contrary, are specific for RA synovial tissue [26, 32]. Moreover, the presence of RA-specific synovial intracellular citrullinated proteins was associated with significantly higher systemic and local ACPA titres [32]. The fact that α-enolase and vimentin are intracellular proteins that can be found in the synovium whereas filaggrin is not present in the synovium and fibrin and CII are extracellular proteins, underlines the relevance of vimentin and α-enolase as antigen in ACPA production. However, it should be noted that vimentin can also be found extracellularly since vimentin is abundant in monocytes and activated macrophages and TNF-α induces the secretion of vimentin from these activated macrophages [33].

Association with SE alleles

SE alleles, which can be considered as a risk factor for the development of RA, are shown to be significantly associated with the presence of antibodies against citrullinated vimentin and not with the presence of antibodies against citrullinated fibrinogen in early-onset RA patients [34]. Remarkably, while SE alleles are known to be associated with ACPAs, no significant effect of the SE alleles on the antibody level towards citrullinated CII was found [8].

It should also be noted that reactivity to CII in RA patients is not restricted to the citrullinated form, in contrast to fibrinogen, α-enolase and vimentin [8, 11, 35]. This shows that citrullination of CII is not crucial to induce reactivity in RA.

Arthritogenic effect and T-cell response to citrullinated proteins

In order to elucidate the identity of the citrullinated antigens that are important in triggering ACPA production, the arthritogenic effect and the T-cell response to citrullinated

proteins should be investigated as well. Hill *et al.* [36] found that citrullinated fibrinogen in DR4-IE transgenic mice was capable of inducing arthritis. Remarkably, these DR4-IE transgenic mice, immunized with citrullinated fibrinogen, also showed Immunoglobulin reactivity to citrullinated vimentin peptides. Moreover, several of these transgenic, immunized mice responded uniquely to citrullinated peptides from vimentin [36]. The polyreactive nature of the antibody response in these mice confounds the interpretation of the potential arthritogenicity of citrullinated fibrinogen [36]. The fact that reactivity to citrullinated vimentin is induced in mice that became arthritic emphasizes an important role of vimentin in this process. However, its precise role still needs to be established. A possible explanation is cross-reactivity between citrullinated vimentin and fibrinogen. Immunizing these DR4-IE transgenic mice with citrullinated vimentin will give more insight into this matter.

Since there is a strong association between ACPA titres and HLA-DRB1 alleles in RA and since ACPA are class-switched antibodies [37], it is interesting to compare the capacity of citrullinated filaggrin, CII, α-enolase, fibrinogen and vimentin to induce T-cell proliferation. The proliferative response of peripheral blood mononuclear cells to both filaggrin and citrullinated filaggrin was rarely observed and did not significantly differ between RA patients and healthy controls [38]. A proliferative T-cell response to native CII was also detected in RA patients [39]. The T-cell response to citrullinated α-enolase still needs to be established. Further research on this topic is necessary to elucidate the role of citrullinated α-enolase in triggering the ACPA response.

Auger *et al.* [40] found that the T-cell proliferative response to citrullinated or native fibrinogen peptides was frequent in RA patients but not in healthy controls. Remarkably, there was no statistically significant difference in T-cell proliferation between citrullinated or native fibrinogen peptides, indicating that citrullination of fibrinogen was not critical to bind HLA-DR and induce T-cell reactivity [40].

In the case of vimentin, on the other hand, citrullination is crucial: a citrullinated vimentin peptide (vim65–77) gave rise to a significantly higher proliferative response and IFN-γ production compared with the unmodified vimentin peptide in immunized HLA-DR transgenic mice [41, 42]. Feitsma *et al.* [42] also found that citrullinated vimentin peptides could be recognized by T cells from $ACPA^+$ $HLA\text{-}DR^+$ patients with RA, whereas the uncitrullinated peptides were not capable of inducing T-cell reactivity [42]. The fact that only the citrullinated form of vimentin induces a T-cell response that can lead to ACPA production and the fact that ACPAs need citrulline to bind, indicates that the binding of ACPAs to both citrullinated and non-citrullinated fibrinogen is not specific and might be due to secondary cross-reactivity. The initiation of ACPA production by citrullinated fibrinogen is also refuted by Steiner [10], who attributed the induction of ACPA production to an antigen different from fibrin, like vimentin, since fibrin deposition occurs after the onset of joint inflammation and ACPAs are present before the onset of the disease.

Taken together, these reports demonstrate an important role of citrullinated vimentin in triggering ACPA production. Therefore, we will focus more in detail on (citrullinated) vimentin and its contribution to the pathogenesis of RA.

Biological function of vimentin

Vimentin is a dynamic intermediary filament, important for the cell structure [43]. The vimentin filaments are involved in the regulation of mechanical stress between chondrocytes and the surrounding matrix tissues [44]. Its assembly and disassembly is regulated by phosphorylation. Other enzymes also impair the polymerization of vimentin filaments: peptidylarginine deiminase (PAD), causes filament disassembly by citrullination of predominantly the non-α-helical head domain [43, 45, 46]. Citrullination of vimentin results in the transformation of a fine vimentin filament network across the whole cell into amorphous clusters situated around the nucleus [43, 45]. This condensation of vimentin at the nuclear periphery may trigger the breakdown of higher order chromatic structures resulting in apoptosis [43, 45]. When peritoneal mouse macrophages are treated with calcium ionophores, which induce apoptosis, selective citrullination of vimentin is observed [43]. Additionally, citrullination of vimentin or its degradation products is also detected in isolated human monocytes and macrophages after ionomycin treatment [47]. It was found that the expression of PAD2 was up-regulated during differentiation of monocytes into macrophages [45]. Vossenaar *et al.* [47] concluded, therefore, that vimentin was citrullinated during apoptosis of long-term-activated macrophages in the inflamed joint and that extracellular proteins like fibrin were citrullinated later in the inflammation cascade, after leakage of PAD from the dying macrophages. However, the assumption that proteins in the inflamed joint are citrullinated during apoptosis is still debated [31]. Indeed, in the inflamed joint, fragmented DNA, which is an indication for apoptosis, is found but no other apoptotic specific morphologies have been detected [31]. In addition, citrullination of other proteins, like histones, is found to be induced in response to inflammatory stimuli and not by treatments that can induce apoptosis [48].

In vitro binding of RA serum to (citrullinated) vimentin

Anti-Sa assay

Vossenaar *et al.* [50] have demonstrated that citrullinated vimentin can establish an RA-specific humoral response. They showed that the anti-Sa antibodies, which are only detected in RA patients, are in fact antibodies against citrullinated vimentin [50].

The importance of anti-Sa antibodies in RA lies in the high specificity of the anti-Sa assay (98–99%) (Table 1) [27, 49, 51]. This was in accordance with Tilleman *et al.* [52] who showed that immune reactivity against

processed isoforms of citrullinated vimentin was almost solely detected in RA anti-CCP$^+$ sera. Because of the high specificity of the anti-Sa test, citrullinated vimentin can be seen as a very important auto-antigen in RA. Like anti-CCP, anti-Sa antibodies were also detected early in disease and could predict the clinical outcome [50, 53]. Moreover, anti-Sa antibodies were more highly associated with RA severity (number of swollen joints, CRP levels, early radiographic erosions) compared with RF, anti-CCP and SE [27, 50, 54] and were considered as the best predictors of severity of recent-onset polyarthritis [55]. Carrier *et al.* [54] conclude that anti-Sa and not anti-CCP is an independent marker of severity in early polyarthritis. Moreover, anti-Sa reactivity at inclusion predicted rapid radiographic damage, even when anti-Sa antibodies later disappeared. This was in contrast with RF and anti-CCP2, which only predicted severe joint damage when these antibodies were persistently expressed [56]. Anti-Sa antibodies also correlated with the presence of HLA-DR SE [50]. In contrast to the citrullinated vimentin, SE alleles were not significantly associated with the presence of antibodies against citrullinated fibrinogen peptide [34]. This was explained by Gyetvai *et al.* [57], who found that not every allele of the SE confers the same risk. Anti-CCP and antibodies against citrullinated vimentin showed the same association pattern (association with Q or D K-RAA and Q or R R-AA), whereas the association pattern for antibodies against citrullinated fibrinogen with SE alleles was completely different. The distribution of Q or D K-RAA and Q or R R-AA alleles was comparable in the anti-citrullinated fibrinogen-positive and -negative patients, explaining the lack of association between anti-citrullinated fibrinogen and SE alleles [57]. In addition, Hill *et al.* [41] showed that citrullinated vimentin peptides bind better to HLA-DR compared with their non-citrullinated counterparts. Unfortunately, they did not compare citrullinated vimentin with other (citrullinated) proteins.

In a study on the antibody response in a North American native (NAN) population, Ioan-Facsinay *et al.* [58] made a comparison of anti-Sa and anti-CCP titres between RA patients and their unaffected relatives. Positive anti-CCP titres were found in RA patients as well as healthy relatives, although with a huge difference between the groups: 91.4 and 19%, respectively [58]. Sixty-one per cent of the ACPA-positive RA patients had anti-Sa antibodies, whereas the ACPA-positive healthy relatives lacked these antibodies. From these data, Menard deduced that RA patients can be divided into two groups: (i) RA patients who behave like healthy subjects and who are anti-Sa negative; and (ii) RA patients who behave like true RA patients and are anti-Sa positive [59]. Taken together, anti-Sa-positive patients can be considered to have a more destructive disease pattern. Rodriguez-Mahou *et al.* [5] considered the anti-CCP2 test as a diagnostic test, whereas the anti-Sa assay has more prognostic value.

Interestingly, it was found that some RA patients who tested negative for anti-CCP appeared to be anti-Sa positive. This leads to the hypothesis that in the anti-Sa test, multiple citrullinated epitopes are present that are different from the epitopes in the anti-CCP test but that are highly specific for RA [50]. Indeed, citrulline on its own is not sufficient, but the amino acids surrounding this citrulline are essential to determine the antigenicity of the epitope [50, 60].

Anti-mutated citrullinated vimentin assay

Although the specificity of anti-Sa is very high, its sensitivity of only 22–43% is a major drawback. Bang *et al.* [61] found an antigenic mutated isoform of vimentin in a human fibroblast cell line that was exposed to oxygen stress. This finding gave rise to a third *in vitro* assay, namely the anti-mutated citrullinated vimentin (anti-MCV) ELISA. This isoform of vimentin was also detected in SF of RA patients. The mutated form of vimentin showed the replacement of several glycine residues by arginine residues [61]. These extra arginines, however, were only occasionally citrullinated, illustrating that not only the citrullination is important, but also that other changes in the protein itself are involved in its antigenicity.

Reports on the sensitivity and specificity of anti-MCV ELISA are rather contradictory. Dejaco *et al.* [3] found that at the high specificity range, the anti-CCP2 assay is more sensitive than the anti-MCV ELISA. Recent studies, on the other hand, showed a higher sensitivity of anti-MCV assay compared with anti-CCP but with a lower specificity [62, 63]. However, it should be noted that the sensitivity of anti-MCV varied according to the disease duration and was found to be 81% in established RA (>2 years duration) and 92% in early RA (<2 years duration). The overall sensitivity and specificity was 84 and 87%, respectively [64]. Similarly, Mathsson *et al.* [65] and Liu *et al.* [66] found that anti-MCV antibodies in early RA showed a higher sensitivity compared with anti-CCP (70.7 *vs* 57.9% and 78.2 *vs* 61.8%), and nearly equal specificities (95 *vs* 96% and 93.4 *vs* 96.3%). The discrepancy between these studies can be explained by the difference in patient population. Mathsson *et al.* [65] and Liu *et al.* [66] focused on early RA ($n = 273$ and $n = 117$), whereas Dejaco *et al.* studied only a limited number of early RA patients ($n = 23$) [3] and Wagner *et al*. [63] only used patients with established RA. Damjanovska *et al.* [62], on the other hand, also studied patients with early RA but these patients had a broader window of disease duration compared with the studies of Mathsson *et al.* [65] and Liu *et al.* [66] (2 years *vs* 12 months). From these reports we can conclude that anti-MCV ELISA is very useful in early RA since higher sensitivity and equal specificities are obtained compared with the anti-CCP assay.

Cross-reactivity experiments between anti-MCV and anti-CCP revealed that only a part of the anti-MCV antibodies react with CCP and vice versa, indicating that anti-MCV and anti-CCP antibodies target different epitopes [67]. Anti-MCV antibodies were also associated with SE and protein tyrosine phosphatase non-receptor type 22 (PTPN22), whereas anti-CCP antibodies were only associated with SE [68, 69].

The diagnostic and prognostic performances of the anti-MCV test were reviewed by Luime *et al.* [70]. They stated that anti-MCV can be used as an alternative for anti-CCP as a diagnostic marker [70]. Qin *et al*. [71] conclude from a meta-analysis on anti-MCV ELISA results that serum MCV may have significant value in the diagnosis of RA. Information on the prognostic performances is limited and contradictory [70]. Some studies showed a stronger correlation of levels of anti-MCV with clinical parameters (ESR, swollen joint count, physician's assessment of disease activity and DAS-28) compared with anti-CCP levels, making anti-MCV a better prognostic marker for future radiographic changes [63, 65, 72]. Others found no correlation between anti-MCV and DAS-28 or additional clinical utility of the anti-MCV test [73, 74].

In vivo binding of RA serum to (citrullinated) vimentin

Besides the above mentioned *in vitro* binding of RA serum to citrullinated antigens it is even more relevant to investigate *in vivo* binding of RA antibodies to (citrullinated) antigens. This *in vivo* binding corresponds to the formation of ICs. ICs can be found in the joint of RA patients, contribute to the pathogenesis of RA [75] and are essential in the initiation of arthritis [76]. Identifying antigens present in these ICs could thus give valuable information on the antigens involved in the initiation of arthritis [76], the destruction of the joint and perpetuation of the inflammation [75]. Zhao *et al.* [77] isolated ICs from plasma by means of C1q ELISA and detected citrullinated fibrinogen in ICs from RA plasma, whereas no citrullinated fibrinogen was found in ICs from healthy plasma. However, it should be noted that it is more relevant to analyse ICs in the joint instead of the periphery since ICs normally accumulate at the site of inflammation and the number of ICs that can escape the joint to get into the circulation is rather small. Additionally, ICs in the periphery are normally rapidly cleared. Therefore, we did not only analyse IC in the periphery but also in the SF. Citrullinated proteins were only detected in IC from SF of CCP+ RA patients and not in IC from RA serum or from SF of CCP- RA patients or SpA patients. Citrullinated fibrinogen was present in IC from SF of only one CCP+ RA patient ($n = 12$), whereas citrullinated vimentin was detected in half of the CCP+ RA patients (6/12). These findings indicate that citrullinated vimentin is the predominant citrullinated antigen in IC from SF of RA patients. This leads to the hypothesis that either: (i) citrullinated vimentin shows a higher affinity with ACPA; or (ii) that ICs with citrullinated vimentin show an insufficient clearance, which could result in a sustained inflammation.

In conclusion, the presence of citrullinated vimentin in the joint, its intracellular localization and the necessity of citrullination of vimentin to bind HLA-DR clearly show the importance of citrullinated vimentin in the pathogenesis of inflammation in RA. The high specificity of the *in vitro* assays to detect citrullinated vimentin (anti-Sa, anti-MCV), the prognostic value of anti-Sa and the high sensitivity of the anti-MCV assay for early RA, also illustrate the critical role of citrullinated vimentin in RA. Additionally, the specific presence of citrullinated vimentin in ICs from SF of CCP$^+$ RA patients further emphasizes the role of vimentin in the pathogenesis of RA.

Rheumatology key messages

- Citrullinated vimentin has an important role in triggering ACPA production and the pathogenesis of RA.
- Antibodies against citrullinated vimentin are especially important in early RA.
- Citrullinated vimentin in ICs of RA SF illustrates its pathological role in RA.

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